ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ (ΟΒΙ)

PCT/ IB 03/ 02785
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ΠΙΣΤΟΠΟΙΗΤΙΚΟ

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Για τον Ο.Β.Ι.
Ο Γενικός Διευθυντής

ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ

Εμμανουήλ Σαμουηλίδης

ΠΑΝΤΑΝΑΣΣΗΣ 5, 151 25 ΠΑΡΑΔΕΙΣΟΣ ΑΜΑΡΟΥΣΙΟΥ - ΤΗΛ.: 61 83 506 - FAX: 68 19 231

ΟΡΓΑΝΙΣΜΟΣ
ΒΙΟΜΗΧΑΝΙΚΗΣ
ΙΔΙΟΚΤΗΣΙΑΣ

ΑΙΤΗΣΗ ΓΙΑ ΧΟΡΗΓΗΣΗ

ΔΙΠΛΩΜΑΤΟΣ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (ΔΕ)
Ή ΔΙΠΛΩΜΑΤΟΣ ΤΡΟΠΟΠΟΙΗΣΗΣ (ΔΤ)
Ή ΠΙΣΤΟΠΟΙΗΤΙΚΟΥ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ
(ΠΥΧ)

1004274/23-6-03

Συμπληρώνεται από τον Ο.Β.Ι.

Αριθμός αίτησης:	20020100335
Ημερομηνία παραλαβής:	16-07-02
Ημερομηνία κατάθεσης:	16-07-02

Με την αίτηση αυτή ζητείται:

☒	**ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)**
☐	**ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:**
☐	**ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)**

Η αίτηση αυτή είναι τμηματική της αίτησης με αριθμό :

ΤΙΤΛΟΣ ΤΗΣ ΕΦΕΥΡΕΣΗΣ :

Σύμπλοκα στεροειδών ορμονών με πρωτεΐνες: Νέες ουσίες για την ειδική ανίχνευση και καταστροφή καρκινικών κυττάρων προερχόμενων από στερεούς όγκους και αιματολογικές κακοήθειες.

ΚΑΤΑΘΕΤΗΣ :

Επώνυμο ή επωνυμία: MEDEXIS AEBE

Όνομα:

Διεύθυνση/Έδρα: Ασκληπιού 4, Κρυονέρι Αττικής, ΕΛΛΑΔΑ

Εθνικότητα: ΕΛΛΗΝΙΚΗ

Τηλέφωνο: **Φαξ:** **E-mail:**

0 Αριθμός	**ΕΠΙΠΛΕΟΝ ΚΑΤΑΘΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ**

ΕΦΕΥΡΕΤΗΣ :

☐ Ο(ι) καταθέτης (ες) είναι ο(οι) μοναδικός(οί) εφευρέτης(ες).

☒ Έντυπο ορισμού του(ων) εφευρέτη(ών) επισυνάπτεται.

ΑΞΙΩΣΕΙΣ:

Αριθμός αξιώσεων: 9

ΔΗΛΩΣΗ ΠΡΟΤΕΡΑΙΟΤΗΤΑΣ

Αριθμός	Ημερομηνία	Χώρα προέλευσης

ΠΛΗΡΕΞΟΥΣΙΟΣ

Επώνυμο: ΔΗΜΟΥ
Όνομα: ΧΡΙΣΤΙΝΑ
Διεύθυνση: ΚΟΥΜΠΑΡΗ 2, 106 74 ΑΘΗΝΑ
Τηλέφωνο: 0103625757 **Φαξ:** 0103626742 **E-mail:**

ΑΝΤΙΚΛΗΤΟΣ

Επώνυμο: ΠΑΠΑΚΩΝΣΤΑΝΤΙΝΟΥ
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Διεύθυνση: ΚΟΥΜΠΑΡΗ 2 106 74 ΑΘΗΝΑ
Τηλέφωνο: 0103626624 **Φαξ:** 0103626742 **E-mail:** EPALAW@HELLASNET.GR

ΔΙΕΘΝΗΣ ΕΚΘΕΣΗ:

☐ Η εφεύρεση παρουσιάστηκε σε επίσημα αναγνωρισμένη έκθεση, σύμφωνα με το ν. 5562/1932, ΦΕΚ 221Α/32.

☐ Σχετική βεβαίωση επισυνάπτεται.

Τόπος: ΑΘΗΝΑ

Ημερομηνία: 16-07-2002

ΥΠΟΓΡΑΦΗ(ΕΣ) ΤΟΥ(ΩΝ) ΚΑΤΑΘΕΤΗ(ΩΝ) ή ΤΟΥ(ΩΝ) ΠΛΗΡΕΞΟΥΣΙΟΥ(ΩΝ) :

ΧΡΙΣΤΙΝΑ Ξ. ΔΗΜΟΥ
ΔΙΚΗΓΟΡΟΣ Α.Μ. 18130
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ΤΗΛ. 36.25.757 - 36.26.624
ΑΦΜ:43953660 - ΔΟΥ: ΙΘ' ΑΘΗΝΩΝ

ΔΗΜΟΥ ΧΡΙΣΤΙΝΑ

ΠΑΡΑΚΑΛΟΥΜΕ Η ΑΙΤΗΣΗ ΝΑ ΕΙΝΑΙ ΔΑΚΤΥΛΟΓΡΑΦΗΜΕΝΗ ΚΑΘΩΣ ΚΑΙ ΤΟ ΟΝΟΜΑ ΚΑΤΩ ΑΠΟ ΤΗΝ ΥΠΟΓΡΑΦΗ. ΣΕ ΠΕΡΙΠΤΩΣΗ ΝΟΜΙΚΟΥ ΠΡΟΣΩΠΟΥ ΝΑ ΔΗΛΩΘΕΙ ΚΑΙ Η ΙΔΙΟΤΗΤΑ ΤΟΥ ΥΠΟΓΡΑΦΟΝΤΟΣ ΓΙΑ ΤΗΝ ΕΤΑΙΡΕΙΑ.

ΟΡΓΑΝΙΣΜΟΣ
ΒΙΟΜΗΧΑΝΙΚΗΣ
ΙΔΙΟΚΤΗΣΙΑΣ

ΟΡΙΣΜΟΣ ΤΟΥ ΕΦΕΥΡΕΤΗ

(ΣΥΜΠΛΗΡΩΝΕΤΑΙ ΣΤΗΝ ΠΕΡΙΠΤΩΣΗ ΠΟΥ Ο ΚΑΤΑΘΕΤΗΣ ΕΙΝΑΙ ΝΟΜΙΚΟ ΠΡΟΣΩΠΟ,
Η Ο ΚΑΤΑΘΕΤΗΣ ΔΕΝ ΕΙΝΑΙ ΚΑΙ ΕΦΕΥΡΕΤΗΣ, Η Ο ΜΟΝΟΣ ΕΦΕΥΡΕΤΗΣ)

Συμπληρώνεται από τον Ο.Β.Ι.

Αριθμός αίτησης:	20020100335
Ημερομηνία παραλαβής:	16 ΙΟΛ. 2002
Ημερομηνία κατάθεσης:	16 ΙΟΛ. 2002

☒	ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)
☐	ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:
☐	ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)

Δηλώνω(νουμε) ως εφευρέτης(ες) στην παραπάνω αίτηση για χορήγηση Ελληνικού τίτλου προστασίας του(τους) :

ΕΦΕΥΡΕΤΗΣ

Επώνυμο: Καστανάς

Όνομα: Ηλίας

Διεύθυνση: Ιατρική Σχολή Πανεπιστημίου Κρήτης, Τ.Θ. 1393, Ηράκλειο, 71110, ΕΛΛΑΔΑ

Ο καταθέτης απέκτησε το δικαίωμα κατάθεσης Ελληνικού τίτλου προστασίας :

- ☒ **Λόγω σύμβασης μεταβίβασης δικαιωμάτων από :** (ημερομηνία)
- ☐ **Λόγω κληρονομικής διαδοχής**
- ☐ **Λόγω συμβατικής σχέσης εργοδότη – εργαζόμενου (υπηρεσιακής ☐ ή εξαρτημένης ☐)**
- ☐ **Με βάση το καταστατικό της εταιρίας**
- ☐ –

0 Αριθμός	ΕΠΙΠΛΕΟΝ ΕΦΕΥΡΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ

Τόπος: ΑΘΗΝΑ

Ημερομηνία: 16-07-2002

ΥΠΟΓΡΑΦΗ(ΕΣ) ΤΟΥ(ΩΝ) ΚΑΤΑΘΕΤΗ(ΩΝ) ή ΤΟΥ(ΩΝ) ΠΛΗΡΕΞΟΥΣΙΟΥ(ΩΝ) :

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ΔΗΜΟΥ ΧΡΙΣΤΙΝΑ

ΠΑΡΑΚΑΛΟΥΜΕ Η ΑΙΤΗΣΗ ΝΑ ΕΙΝΑΙ ΔΑΚΤΥΛΟΓΡΑΦΗΜΕΝΗ ΚΑΘΩΣ ΚΑΙ ΤΟ ΟΝΟΜΑ ΚΑΤΩ ΑΠΟ ΤΗΝ ΥΠΟΓΡΑΦΗ. ΣΕ ΠΕΡΙΠΤΩΣΗ ΝΟΜΙΚΟΥ ΠΡΟΣΩΠΟΥ ΝΑ ΔΗΛΩΘΕΙ ΚΑΙ Η ΙΔΙΟΤΗΤΑ ΤΟΥ ΥΠΟΓΡΑΦΟΝΤΟΣ ΓΙΑ ΤΗΝ ΕΤΑΙΡΕΙΑ.

Steroid-Protein conjugates: New compounds for the selective identification and elimination of tumor cells derived from solid cancers and hematological malignancies

Classical steroid hormone action is mediated through intracellular steroid hormone receptors. These proteins dimerize, after steroid binding, translocate to the nucleus, and exert specific nuclear transcription factor effects on specific steroid-sensitive genes [1]. In recent years, however, a number of studies indicate that, in addition to the above genomic action, steroids bear non-genomic effects, mediated in minutes, and implicating different pathways than those involved in classical steroid receptor action [2, 3]. Non-genomic steroid actions were, in addition, been found in cells not expressing classical steroid receptors. The above, non-genomic steroid receptor actions were attributed to another class of steroid receptors, found on membrane of cells, and being biochemically, immunologically and pharmacologically different from classical steroid receptors. Until now, non-genomic steroid effects were found for estradiol, cortisol, and testosterone, in animal tissues, usually not- expressing classical receptors [2-13]. Activation of these non-classical steroid sites, found on membranes of cells was the increase of the flux of extracellular calcium to the cytosol [8-10, 12, 14], and in some times, modifications of the cytoskeleton [7, 15]. In all cases, BSA-conjugated steroids were used as ligands for these extracellular (membrane) steroid sites, in order to identify these sites. Indeed, covalent binding of steroids with high (60 kD) molecular weight proteins makes these molecules to loose their lipid solubility (and therefore the property of translocating to the cell through the plasma membrane) and confines them with water solubility and the possibility of binding to specific steroid sites. Commercially available sources of these compounds are currently available (e,g, Sigma Chemical Co. St Louis, MO, USA). Nevertheless, human applications of these membrane steroid receptor activation have not been described so far.

The invention consists of the development of membrane steroid receptors agonists, determined by the association binding of these molecules with membrane steroid receptor bearing cells.

The invention will use these molecules for the selective identification and elimination of cancer cells derived from solid tumor and hematologic malignancies in humans.

The specific molecules will be used, according to the invention, for the production of diagnostic and therapeutic agents. They are protein-conjugated (BSA-conjugated, Human Serum Albumin (HSA)-conjugated, binders or antibodies of selective human tumoral cell antigens for example –the list is not exclusive-) steroids.

By the term steroids are described all natural and synthetic steroid hormones, their analogs and conjugates like their sulphate- or fatty acid-esters, their precursors and metabolites or their analogs (steroid or non steroid in structure).

As analogs the inventors claim all natural, semisynthetic or synthetic polycyclic molecules, capable to bind to human membrane steroid receptors, their mixtures, precursors and metabolites.

The invention will be used, as illustrated in the examples provided, to produce specific diagnostics in cases of solid tumors and hematological malignancies.

The action of membrane steroid receptors, as illustrated by the examples, being the modifications of actin cytoskeleton, and the potentiation and extension of the action of cytoskeleton-acting drugs (eg. Taxol®) makes membrane steroid receptor agonists an interesting class of potential drugs.

The inventors will therefore target the production of new drugs, capable for a specific and selective binding to a class of membrane steroid receptors, present, as illustrated in the provided examples, in selective malignancies, in view of a selective primary or adjuvant chemotherapy. In another aspect, these agents, used as chemotherapeutics, could be used, alone or in association with other chemotherapeutics (ex. Taxol® or equivalent drugs), in order to prevent, or modulate the chemoresistance of selective tumors.

The inventors will determine the best mode of administration of these drugs (local or general, injectable or locally applied during interventions, etc).

In order to increase the selectivity of the steroid-protein conjugates, different attachments of the steroid moiety to the protein will be made (ex. attachment trough addition of an acid group at carbon positions 1, 3, 7, 11 or 15 –the list is not exclusive-). The covalent attachment of the steroid moiety to proteins will be made by the use of conventional methods (ex. attachment of a carboxy-methyl ether moiety, and the attachment to proteins by the action of carboxydiimide).

Other characteristics and advantages of the invention are given in the following description, the References and the attached Figures.

- Figure 1 presents the binding and selectivity characteristics of membrane testosterone receptors in LNCaP cells.
- Figure 2 shows the detection of membrane testosterone receptors, in LNCaP cells by flow cytometry (left panel) and confocal laser scanning microscopy (right panel).
- Figure 3 shows the detection of testosterone membrane receptors (by flow cytometry) in cases of prostate cancer, benigh prostate hyperplasia (BPH) and peritumoral non-tumor cells.
- Figure 4 presents the detection of membrane testosterone receptors in touch preparations of prostate tumors (prostate cancer at the left and BPH at right) and histological slides of prostate intraepithelial neoplasia (PIN, at left) and prostate cancer (right).
- Figure 5 presents the detection of estrogen, progesterone and androgen receptors in ER positive (upper lane) and ER negative breast cancer.
- Figure 6 shows the detection of testosterone, estrogen and progesterone receptors in bone marrow CD34 and AC133 cells (upper and lower lanes).
- Figure 7 shows the modification of actin cytoskeleton in LNCaP cells by testosterone-BSA, assayed by confocal scanning laser microscopy (upper panels) and the increase of polymerized actin by testosterone-BSA, assayed by biochemical methods (lower panel).
- Figure 8 shows the modification of cell viability by a24 hour incubation of testosterone-BSA, alone or associated with Taxol® (A), and the effect after an

additional incubation of 48 hours, in the absence of drugs (B). Panel C presents the dose-response of cells to testosterone-BSA alone or additioned with Taxol®.

Material and Methods

Cell line

The human prostate cancer LNCaP cell line, originally isolated from a lymph node metastasis of prostate adenocarcinoma [16], was purchased from DSMZ (Braunschweig, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air. They were subcultured once a week and incubated in serum-free medium for 24 h before any experiment. All culture media were purchased from Gibco BRL (Life Technologies, Paisley, UK).

Cell number was assayed using the tetrazolium salt assay [17]. Cells were incubated for 3h at 37°C with the tetrazolium salt (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide, Sigma, St Louis, MO). Living cells reduced the dye to purple formazan seen as dark blue crystals. At the end of the incubation period they were dissolved with propanol-1 and the absorbance was measure at 575 nm, within one hour.

Detection of membrane androgen receptors

i. Binding assays

Membrane preparation

Cells, cultured in 150 cm^2 flasks without serum, were washed twice with phosphate-buffered saline (PBS), removed by scraping and centrifuged at 1500 rpm. Pelleted cells were homogenized by sonication in 50mM Tris-HCl buffer pH 7.4 containing freshly added protease inhibitors (10µg/ml PMSF and 1µg/ml aprotinin). Unbroken cells were removed by centrifugation at 2500g for 15min. Membranes were obtained by centrifugation at 45,000g for 1 hour, and washed once by the same buffer. Protein concentration was measured by the method of Bradford [18].

Binding conditions

Saturation binding experiments were performed in a final volume of 0.1 ml, containing cell membranes at a final protein concentration of 2mg/ml and at least 6 different concentrations of [^{3}H]testosterone (ranging 2-50 nM) without (total binding) or with (non-specific binding) a 1000-fold molar excess of unlabelled androgen (DHT). For displacement binding experiments, cell membrane preparations at a final concentration of 2mg/ml were incubated with 5nM of [^{3}H]testosterone (specific activity 95 Ci/mmole, Amersham-Pharmacia, Buckinghabshire, UK) in the absence or in the presence of different concentrations of un unlabelled steroid (DHT, estradiol, progesterone, all from Sigma, St Louis, MO), ranging from 10^{-12} to 10^{-6} M. Non specific binding was estimated in the presence of 5μM DHT. In both types of binding experiments, after an overnight incubation at 4°C, bound radioactivity was separated by filtration under reduced pressure through GF/B filters previously soaked in 0.5% polyethylenimine (PEI) in water and rinsed three times with ice-cold Tris-HCl buffer. Filters were mixed with 4 ml scintillation cocktail and the bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard) with 60% efficiency for Tritium.

ii. Flow cytometry

LNCaP cells, cultured in serum free medium for 24 hrs, were detached from the culture flask by scraping and suspended in PBS at a density of 10^6 cells/ml. They were incubated at room temperature with 10^{-7} M testosterone-BSA-FITC conjugate for different periods of time (1 min to 1 hour). A thousand-fold BSA-FITC was used to determine non-specific binding. Cells were analyzed by flow cytometry using a Coulter Epics XL-MCL apparatus (Beckman-Coulter Inc. Foullerton CA, USA) in a sample size of 10,000 cells gated on the basis of forward and side scatter. Testosterone3-(O-carboxymethyl)oxime – BSA-FITC (named testosterone-BSA-FITC), testosterone3-(O-carboxymethyl)oxime – BSA (named testosterone-BSA), estradiol6-(O-carboxymethyl)oxime – BSA-FITC (named estradiol-BSA-FITC), progesterone3-(O-carboxymethyl)oxime – BSA-FITC (named progesterone-BSA-FITC) and BSA-FITC were obtained from Sigma (St Louis, MO).

iii. Confocal Laser microscopy

LNCaP cells were allowed to grow on poly-L-lysine coated glass coverslips for at least 48 hours before culture medium was replaced with serum free medium. After a 24-hour period, cells were washed twice with PBS and incubated with Testosterone-BSA-FITC for 30 min in the presence or in the absence of DHT. As a negative control BSA-FITC was used. Cells were then washed twice with PBS and fixed with 2% PFA in PBS for 30 min. Coverslips were mounted on to slides using a 1:1 (v/v) mixture of glycerol and Vestashield (Vector, Burlingame, CA). Specimens were analysed using a confocal laser scanning microscope (CLSM) (Leica TCS-NT, Lasertechnik, Heidelberg, Germany).

Detection of membrane steroid receptors in paraffin-embedden tissue preparations

Tissue slides were prepared from paraffin blocks of formalin fixed tissue preparations. Three-four micron (μm) thick tissue sections were cut and put on on SuperFrost Plus slides (Kindler O GmbH, Freiburg, Germany), incubated at 56°C for 2h, washed six times with xylene (5min each), followed by 96%, 80% and 70% ethanol for five minutes each, and finally with distilled water for 20 min. Tissue slides were then incubated in citrate buffer in a microwaves oven at 500 Watts, three times for 4.5 minutes each, washed in distilled water and Tris buffered saline (TBS, 10 mM, pH 7.4). Non-specific absorption of BSA was eliminated by a 10 min incubation with a 2% solution of BSA in TBS, followed by two washes with TBS. Slides were then incubated for 10 min with BSA-FITC conjugated steroids and washed with TBS. Coverslips were mounted on to slides using a 1/1 (v/v) mixture of glycerol and Vestashield (Vector, Burlingame, CA). Specimens were analysed using a confocal laser scanning microscope (CLSM) (Leica TCS-NT, Lasertechnik, Heidelberg, Germany).

Determination of monomeric and polymerized actin

For measurements of the monomeric (Triton soluble) and polymerized (Triton insoluble) actin, LNCaP cells were incubated for 10 min with or without DHT or testosterone-BSA (10^{-7} M). Then, 500 μl of Triton-extraction buffer (0.3% TritonX-100, 5 mM Tris, pH 7.4, 2 mM EGTA, 300 mM sucrose, 2 μM phalloidin, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM sodium orthovanadate, and 50 mM NaF) were added, and the mixture was incubated for 5 minutes on ice. After removing the buffer, soluble proteins were precipitated with equal volumes of 6%

PCA. The Triton-insoluble fraction remaining on the plate was precipitated with 1 ml of 3% PCA. Equal volumes of each fraction were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting protein-bands were transferred onto nitrocellulose membrane, and the membrane was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1h at room temperature. Antibody solutions (in TBS-T) were added for 1h at room temperature [monoclonal mouse anti-actin first antibody (Amersham-Pharmacia, Bukinghamshire, UK) and second horseradish peroxidase-coupled antibody (Chemicon, Temecula,CA)]. Blots were developed using the ECL system (Amersham-Pharmacia, Bukinghamshire, UK) and the band intensities were quantitated by PC-based image analysis (Image Analysis Inc., Ontario, Canada) [19].

Immunoprecipitation, kinase assays and immunoblotting analysis

Testosterone-BSA or DHT-treated, as well as untreated (control) cells were washed three times with ice-cold PBS and suspended in cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris pH 7.4 and 137 mM NaCl, supplemented with protease and phosphatase inhibitors. Cleared lysates were preadsorbed with protein A-Sepharose for 1 h at 4 °C, centrifuged and the supernatants (equal amounts of protein) were subjected to immunoprecipitation using the indicated antibodies and the protein A-Sepharose beads.

The lipid kinase activity of PI-3 kinase was measured by the method of Auger *et al* [20] with minor modifications. Protein A-Sepharose beads containing immunoprecipitated phosphotyrosine proteins were washed three times with Buffer A (20 mM Tris pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Nonidet P-40, 0.1mM Na_3VO_4), three times with 5 mM LiCl in 0.1 M Tris (pH 7.4) and twice with TNE (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM Na_3VO_4). The immunoprecipitates were then resuspended in TNE and the PI-3 kinase activity was assayed using 0.2 mg/ml phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) as a substrate, in the presence of 58 μM ATP, 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol) and 14 mM $MgCl_2$, for 10 min at 37 °C. The reaction was stopped by the addition of 1 M HCl and methanol/chloroform (1/1). After mixing vigorously and centrifuging to separate the phases, the lipids in the organic lower phase were separated by TLC on

oxalated silica gel 60 sheets, as described [21]. Chromatographed lipids were also visualized by iodine staining and compared to the migration of known standards.

For immunoblot analysis, the cell lysates or the immunoprecipitates were suspended in Laemmli's sample buffer and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane, and the membrane was blocked with 5% nonfat dry milk in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1h at room temperature. Antibody solutions (in TBS-T containing 5% nonfat dry milk) were added overnight at 4 °C (first antibody) and for 1h (second horseradish peroxidase-coupled antibody). Blots were developed using the ECL system and the band intensities were quantitated by PC-based image analysis (Image Analysis Inc., Ontario, Canada).

Affinity precipitation

Affinity precipitation with GST-PBD was performed using an assay based on the method of Benard *et al* [22]. Cells were lysed in Mg^{2+} lysis buffer (MLB), that was provided by the assay kit (UBI, Lake Placid, NY), were mixed with 8 μg GST-PBD bound to glutathione-Agarose and incubated for 1h at 4 °C. Precipitates were washed three times with MLB and suspended in Laemmli's sample buffer. Proteins were separated by 11% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with anti-Cdc42 or anti-Rac antibody.

Results

Membrane androgen binding sites on the human prostate cancer cell line LNCaP

Membranes, prepared from cultures of LNCaP cells were incubated with different concentrations of [^{3}H]testosterone (ranging 2-50 nM) without (total binding) or with (non-specific binding) a 1000-fold molar excess of unlabelled androgen (DHT). After overnight incubation at 4°C, membrane-bound radioactivity was separated and counted. It was found, as presented in Figure 1A, that [^{3}H]testosterone, ranging from 1 to 50 nM, induces a specific saturable binding. Scatchard analysis of the results (Figure 5A insert) revealed a high binding affinity for testosterone (K_D 10.9 nM) and

a number of binding sites of 144.3 fmoles/mg protein, corresponding to an approximate number of 13340 sites/cell.

The androgen selectivity of this membrane-binding component was verified by competition displacement experiments. Membranes were incubated with [^{3}H]testosterone in the presence of varying concentrations of DHT or other steroids (10^{-12}-10^{-6} M). As shown in Figure 1B, DHT produced a displacement of radiolabeled testosterone. In contrast, estradiol and progesterone displaced radiolabelled testosterone with a significant lower affinity (10^4- and 10^2-fold respectively) confirming the androgen selectivity of the identified membrane-binding site.

The presence of membrane testosterone receptors was equally identified using the testosterone analog testosterone3-(O-carboxymethyl)oxime – BSA-FITC, obtained from Sigma (St Louis, MO). This analog is not capable to penetrate the cells, because of a covalent attachment of the steroid with BSA. As shown in Figure 2, left panel, a specific membrane binding of testosterone-BSA was found, by flow cytometry, on membranes of LNCaP cells. The association of testosterone-BSA with membrane receptors was observed at 1 min, was maximal after 10 min, and remained unchanged after 30 min of incubation. The membrane binding was equally verified by confocal laser microscopy, as shown in the right panel of Figure 2. As shown, only membrane staining was found by the use of the testosterone-BSA conjugate, rulling out the hypothesis of a possible internalization of the compound.

From these experiments it was concluded that prostate cancer cells possess specific, high affinity membrane binding sites, which are selective for androgens.

Identification of membrane testosterone receptors in specimens of prostate cancer.

In a series of 14 prostate cancer specimens, 10 transurethral resections for benign prostate hyperplasia (BPH), and 8 microscopically verified non-malignant specimens from the same cases, we have prepared epithelial cell specimens. Cells were immunostained with monoclonal antibodies to vimentine, cytokeratine and PSA, to evidence stromal, and normal or malignant epithelial cells respectively. It was verified that epithelial cells accounted for more than 85% of total cells, in all studied

specimens. Cells were incubated for 10 min with testosterone-BSA and assayed by flow cytometry. As shown in Figure 3, membrane testosterone binding was very low in cases of BPH, while a high binding was found in all cases with cancer. In this respect, membrane testosterone receptors can fully discriminate malignant from benign cases of prostate tumors.

The above discrimination was apparent also by fluorescent staining of prostate epithelial cells in touch preparations (Figure 4, upper panel). Indeed, after surgery, gross identified malignant lesions of the surgical preparations were touched on SuperFrost/Plus slides, adhered cells were stained with testosterone-BSA-FITC, and immediately analyzed in a fluorescent microscope. As shown, only malignant epithelial cells were stained, while BPH epithelial cells presented a very low fluorescence. Finally, as shown in the lower panel of Figure 4, membrane testosterone receptors can be identified in routine histological slides, from formalin-fixed, paraffin-embeded cases of prostate cancer. It is interesting to note further, that testosterone membrane staining can identify cases of intraepithelial neoplasia specifically.

From the above results, it becomes apparent that testosterone membrane receptors are a specific and selective element of prostate cancer.

Identification of membrane steroid receptors in breast cancer specimens

Estrogen- progesterone- and androgen-membrane binding was assayed in steroid receptor positive and negative tumors, as assayed by immunocytochemistry. Typical results are presented in Figure 5. As shown, regardless of the state of intracellular steroid receptors, BSA-conjugated steroids identify components in histological preparations. Androgen receptors are present in low concentrations in these breast tumors. In contrast, estradiol-BSA and progesterone-BSA identify pericellular components in tumoral cells in the breast. This is more obvious in ER/PR negative tumors, in which there is no interaction with intracellular receptors. Indeed, in ER/PR positive cases, as there is a cellular damage, during slide preparation, these exist, in some cases, a diffuse pattern of staining, which, in some cases can not be attributed to a pericellular, intracellular, or nuclear binding.

Identification of membrane steroid receptors in hematological malignancies

In normal blood white blood cells (WBC) we have identified testosterone membrane binding (performing routine flow cytometric assays). The distribution of testosterone-positive cells are shown in Table 1.

Table 1: Distribution of membrane testosterone positive (Testo+) cells in different groups of WBC in 20 health blood donors.

	Lymphocytes	**Monocytes**	**Polymorphonuclear**
% of total WBC	35.6±1.03	7.7±0.41	56.1±1.18
Testosterone +	8.9	2.2	11.6
%Testo+ in category	**23**	**28**	**20**

As shown, membrane testosterone receptors are expressed in all three classes of WBC. It is interesting that monocytes express, in higher percentages these sites.

The analysis of testosterone membrane receptor in subclasses of lymphocytes is expressed in Table 2.

Table 2: Distribution of membrane testosterone positive cells in different categories of lymphocytes. T cells were assayed by the assay of CD3 marker, B by the expression of CD19, and NK cells by the expression of CD56 lymphocyte antigen. Coexpression of the above marker (marked by a PE-labeled monoclonal antibody) with testosterone-BSA-FITC was used for the detection of the testosterone positive subset of cells.

	T	**B**	**NK**
% of total Lymphocytes	76	14	9.7
Testosterone +	18	4.25	3.4
%Testo+ in category	**23**	**30**	**34**

As shown, B-lymphocytes and NK cells express preferentially the testosterone receptor, as compared to T-lymphocytes. In addition, further analysis of T-cells, showed an equal distribution in CD4 and CD8 positive lymphocytes.

In four cases of malignancies, the distribution of testosterone membrane receptor is shown in Table 3.

Table 3: Detection of membrane testosterone binding in four cases of hematological malignancies. The mean of the normal controls is given for comparison.

Diagnosis	Lymphocytes		Monocytes		Polymorphonuclear	
	%	% Testo+	%	% Testo+	%	% Testo+
ALL remission	19	24	8	25	73	19
ALL	81	16	1	25	18	14
AML Remission	32	24	10	35	58	27
Malignant Lymphoma	70	19	7	34	23	16
Normal	36	25	8	28	57	21

As shown, membrane testosterone binding was found decreased in lymphocytes in ALL, while it returns to the levels of normal controls in remission. In contrast, in malignant lymphma and AML, increased testosterone membrane binding is found in monocytes, while in ALL and the case of lymphoma studied, polymorphonuclear membrane testosterone receptors are found to be decreased.

In Table 4, it is presented the distribution of testosterone receptors in different subclasses of lymphocytes. As shown, membrane testosterone receptors are equally low in all three subsets of lymphocytes, in the case of ALL, returning to normal values after remission. The same result is also found in the case of AML.

Table 4: Distribution of membrane testosterone receptors, in four cases of hematological malignancies. Results obtained in normal blood donors are given for comparison.

Diagnosis	T		B		NK	
	%	% Testo+	%	% Testo+	%	% Testo+
ALL remission	85	20	35	6	10	3
ALL	64	9	5	2	2	0.2
AML Remission	73	13	5	1	15	5
Malignant Lymphoma	90	17	13	4	5	1
Normal	76	18	14	4	10	--3

It is interesting to note that the distribution of testosterone membrane sites shows a differential distribution in normal and leukemic cells. In addition, as shown in Figure 6, bone marrow stem cells (both CD34 and AC133 positive) express membrane binding sites for all three steroids tested (estrogen, progesterone and androgen). It is

therefore possible that the expression of non-mature lymphoid cells account for the differential expression of membrane testosterone receptors, and therefore, the invention could be also used for the detection and treatment of hematological malignancies.

Interaction of membrane steroid receptors with actin cytoskeleton

Figure 7 shows the effect of action of testosterone-BSA conjugate to the actin cytoskeleton of LNCaP human prostate cancer cells, assayed by confocal laser scanning microscopy. As depicted, 10 minutes after testosterone application, a profound modification of the cytoskeleton occurs. Actin filaments are redistributed at the periphery of the cell, while, as presented at the lower panel of Figure 7, the significant decrease of the ratio of soluble (monomeric) to insoluble (polymerized) actin indicates that profound alterations of the actin cytoskeleton occur, in favor of a polymerization prossess.

Further work revealed that testosterone receptors located on cell membranes of LNCaP cells activate key signaling molecules in a hierarchy of FAK→PI-3 kinase→ Cdc42/Rac1→actin reorganization. The fact that testosterone was less active than testosterone-BSA conjugate further indicates that this signaling cascade might be specific of the activation of testosterone membrane binding sites. These results outline, for the first time, a signal transduction pathway that was triggered by membrane testosterone receptors in prostate cancer cells and leads to actin reorganization.

Long term (24 hours) incubation with testosterone-BSA decrease cell proliferation of cancer cells

In view of the above results, we incubated prostate cancer LNCaP cells with testosterone-BSA alone (10^{-7} M) or together with Taxol® (10^{-8} M) for 24 hours. As shown in Figure 8A, a 50% decrease of cells incubated with testosterone –BSA alone was found. In adition, a potentiation of the action of Taxol® by ~7% was also found. If medium was replaced after this 24 hours incubation, and cells were provided with fresh medium without any added substance and let to stay for additional 48 hours (a condition mimicking the weekly administration of low doses of Taxol® in clinic),

cells recover partially (Figure 8B). In this case, the action of testosterone-BSA is more potent than that of Taxol®. This effect is dose-related to the testosterone-BSA, as shown in Figure 8C, indicating an additive effect of testosterone-BSA with taxol®.

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Claims

1. Non-toxic compounds, capable to bind to membrane steroid binding sites.
2. New compounds synthesized by the association of steroid hormones with serum proteins, binders or antibodies of selective human tumoral cell antigens, according to claim 1.
3. Compounds synthesized according to claims 1 and 2, with different attachment to the steroid molecule.
4. Compounds elaborated according to claims 1, 2 and 3, with analogs of steroids.
5. Use of compounds according to claims 1, 2, 3 and 4, as cytoskeletal modifying agents.
6. Use of compounds, according to claims 1, 2, 3, and 4 for the production of specific diagnostics, for solid cancers or hematological malignancies.
7. Use of compounds, according to claims 1, 2, 3 and 4 for the production of drugs, for the treatment of solid cancers or hematological malignancies.
8. Use of compounds, according to claim 7, as adjuvant or associated chemotherapy with other cytoskeletal modifying agents (ex. Taxol®), in solid tumors or hematological malignancies.
9. Use according to claims 7 and 8 of the best form of administration of these drugs (general or local chemotherapy).

Summary

The invention consists to the development and use of steroid hormone (and their analogs)-protein conjugates characterized by their binding to membrane steroid receptors on cells derived from solid and hematological malignancies, and, through the modification of cytoskeleton, capable to induce and/or enhance the cytotoxic action of cytoskeleton modifying agents. The invention is particularly useful for the fighting against malignant diseases, by providing selective and specific agents capable to identify and modify cells expressing a malignant phenotype.

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